Microbiology for chemical engineers – from macro to micro scale

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ABSTRACT: Recent developments in microbial techniques (such as PCR, GE, FISH) have allowed researchers to detect, identify and quantify microorganisms without the limitation of culture-dependent methods. This has given both engineers and scientists a more fundamental understanding about systems containing microorganisms. These techniques can be used to monitor bacteria in wastewater treatment systems, soil and sea, industrial fermentation, food technology, and improve floccability, etc. However, despite these techniques being readily available and relatively cheap, they are not widely used by engineers. Hence, the aim of this paper is to introduce these techniques, and their applications, to chemical engineers. Two different studies related to industrial wastewater treatment, but applicable to general microorganism systems, will be presented: (1) microbial stability of pure cultures, and (2) bioreactor population shifts during alternating operational conditions. In (1), two bioreactors, inoculated with two different pure cultures, (A) *Xanthobacter aut* GJ10 and (B) *Bulkholderia sp* JS150, degrading 1,2-dichloroethane (DCE) and monochlorobenzene (MCB), respectively, were followed over time (Emanuelsson *et al*., 2005). Specific and universal 16S rRNA oligonucleotide probes were used to identify the bacteria. It was found that bioreactor (A) remained pure for 290 days, whereas bioreactor (B) became contaminated within one week. The difference in behaviour is attributed to the pathway required to degrade DCE. In (2), the stability of a bacterial strain, which was isolated on the basis of its capability to degrade 2-fluorobenzoate from contaminated soil, in three different, up-flow fixed bed reactors operated under shock loads and starvation periods, was followed by denaturing gradient gel electrophoresis (DGGE) (Emanuelsson *et al*., 2006). All bioreactors were rapidly colonised by different bacteria; however, the communities remained fairly stable over time, and shifts in bacterial populations were mainly found during the starvation periods.

KEYWORDS: bacteria; stability; non-sterile; long-term; bioreactor

INTRODUCTION

Recent advances in microbiology have allowed comparison between different microorganisms through analysis of a particular sequence of the DNA – for example the ribosomal RNA (rRNA).

This has shown that all cellular life belongs to three domains: bacteria, archea and eukarya (Woese *et al*., 1990). Following this, it has been shown that some parts of these sequences are conserved throughout all organisms, some being group-specific and some species-specific (Head *et al*., 1998). By studying the diversity in the genes, it is possible to compare similarities between organisms, and from this information to create phylogenetic trees, determining how closely related different species are, and to identify them (Head *et al*., 1998; Hugenholtz *et al*., 1998). In practice, any gene could be used as a molecular marker, but the rRNA gene is advantageous because it is not transferred horizontally, its domain structure contains both conserved and variable regions, its concentration is high in the cell and it is present in all microorganisms (Muyzer and Ramsing, 1995; Head *et al*., 1998).

It is relatively easy for a microbiologist to determine the specific rDNA sequence of a species, and once this is known, it can be compared to already known sequences from other species. Over 200 000 rDNA gene sequences have been published on the web (http://rdp.cme. msu.edu), and therefore a good reference library is available both to compare new sequences and to find specific sequences. This information can then be used by microbiologists and environmental, chemical and biochemical engineers, to follow either whole populations or individual strains over time.

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METHODOLOGY

There are several different ways of detecting and identifying bacteria in either pure or mixed samples. The methodology is generally chosen on the basis of the application. The methods can firstly be divided into two groups: (1) where the DNA/RNA is firstly extracted and thereafter generally amplified, and (2) where whole cells are used and the DNA/RNA is not amplified (Lipski *et al*., 2001).

For each methodology, the first step is to design a genetic marker. This is a short sequence of the DNA/RNA bases complementary to the sequence it is designed to detect. The choice of the marker determines the application. For example, to detect all bacteria in a sample, a general sequence is required, but to detect a specific species, a sequence that is present only in this particular species is required. Sequences that are designed to amplify DNA/RNA are called *primers*, and sequences that attach permanently for direct visualisation of bacteria are called *probes*. When the primers or probes are applied to the sample, they will hybridise (bind) to the specific sequence in the DNA they are designed to match with. Many sequences are either published in journals or on the web. If a desired sequence has not been previously published, a probe design program, for example Primrose, can easily be used to design them. Some of the more common techniques used in the process of identifying or characterising bacteria are described below. For more details, see the review by Lipski *et al*. (2001).

Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique that amplifies a specific DNA sequence. It uses two primers complementary to either side of the piece of DNA to be amplified. The total DNA sequence is first denatured (i.e. it is made single-stranded by increasing the temperature). The temperature is then decreased, allowing the primers to bind to a matching denatured sequence. DNA polymerase (an enzyme) then extends the DNA sequence, starting from the bound primers. The procedure is thereafter repeated several times until millions of copies have been created. There are several versions of PCR; for example, nested PCR can be applied to increase specificity and RT-PCR can be used to quantify mRNA. Generally, PCR requires a further analysis step, such as gel electrophoresis (GE).

Gel electrophoresis

GE can be used to visualise PCR products (and other DNA samples). The basic principle of GE is that the DNA sequences are separated on the basis of how fast they migrate in an electric field. The speed of migration depends on physical properties of the DNA, such as size, secondary structure and charge. By staining the gel – with for example ethidium bromide – the amplified DNA is made visible with UV-light. For example, von Canstein *et al*. (2002) applied PCR and GE to study the microbial diversity in a packed-bed bioreactor exposed to mercury.

However, there are more sophisticated versions of GE, for example, denaturating/temperature gradient gel electrophoresis (D/TGGE). The separation is based on the electrophoretic mobility of the PCR-amplified fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (a mixture of urea and formamide), or increasing the temperature gradient. Once a fragment reaches its melting point, it stops. This allows sequences with the same length but with different base-pair sequences to be detected, because of the highly specific melting and denaturing points for every sequence. For example, Kaewpipat and Grady (2002) used it to analyse sequencing batch reactors, and Eichner *et al*. (1999) used it to study the microbial diversity in an activated sludge system bio-augmented with engineered bacteria exposed to phenol shock loads. Readers are referred to Muyzer and Smalla (1998) for a detailed review of applications of D/TGGE.

Another version is single-stranded conformation polymorphism (SSCP). SSCP takes advantage of the instability of single-stranded DNA. In the absence of a complementary strand, base pairing might result in loops and folds that give the single strand a unique 3D structure, regardless of its length. Even a single nucleotide affects the strand's mobility through a gel by altering its 3D conformation (Sunnucks, 2000). The DNA sequence of interest is first amplified by PCR and thereafter denatured before analysis. For example, Zumstein *et al*. (2000) used SSCP to analyse community dynamics in an anaerobic digester, and Dabert *et al*. (2001) applied SSCP to monitor the impact of inoculating a phosphorous-acclimatised sludge in a bioreactor for phosphorous removal.

Amplified ribosomal rDNA restriction analysis (ARDRA)

When using ARDRA, the DNA is first extracted and a specific sequence of the rDNA, which is variable between bacteria, is amplified by PCR. Each amplified sample is thereafter cut up into small sequences by a restriction enzyme. A restriction enzyme is an enzyme that cuts DNA at specific sites, and the enzyme recognises a few base pairs in the DNA and will cut the DNA at all places where this short sequence of base pairs is present; since each bacteria has a unique DNA

sequence, each sequence amplified will be cut in a different way. The short sequences are then run on GE, where individual bacteria give rise to a specific pattern. One drawback with this technique is that each bacterium needs to be isolated before restriction with the enzyme. For example, Massol-Deya *et al*. (1997) determined the variation in dominant species in fluidised bed reactors, Fernandez *et al*. (1999) analysed population shifts in methanogenic reactors, and Smit *et al*. (1997) determined community shifts in copper-contaminated soil using such technique.

Fluorescence *in situ* hybridization (FISH)

Different from PCR-based methods, in FISH the probe is used to identify whole cells. The hybridisation is applied to morphologically intact cells (*in situ*); hence no extraction of DNA/RNA is necessary. A probe that is labelled with a fluorochrome (fluorescent dye) is used. The probe is generally designed to match a 16S rRNA sequence, but in theory it can be designed to match any gene. The probe hybridises (binds) with the complementary sequence found in the cells. Cells with a probe bound to them will fluoresce under a fluorescence microscope, while cells without a probe will not fluoresce. This allows identification, detection and quantification of any specified bacteria. Several probes can be used together, and by varying the specificity of the probes all bacteria present, as well as specific bacteria, can be detected simultaneously. Furthermore, the morphology of the cells and their spatial resolution can be determined as the sample is analysed *in situ*. FISH has, for example, been used to study the community of a sand filter (Bouchez *et al*., 2000) and to follow a bio-augmented bacterium (Neef *et al*., 1996).

Dot-blot hybridisation

Dot-blot hybridisation also uses fluorescent-labelled probes, but instead of being applied to intact cells, the DNA/RNA is extracted from cell samples and subsequently fixed on a membrane (amplified or not) in which the hybridisation takes place. As in FISH, quantification is easy because the signal intensity of the probe corresponds to the concentration in the sample. It has, for example, been used to identify methanogens (Raskin *et al*., 1994) and to detect genes coding for de-nitrification and nitrification (Kloos *et al*., 1995).

Comparison of methods

A drawback with PCR-based methods in comparison to FISH and dot-blot hybridisation is that it is complicated to quantify the bacteria because of biases in PCR, cloning and cell lysis (Head *et al*., 1998; Fernandez *et al*., 1999). A major drawback with FISH and dot-blot is that only bacteria that have a probe designed for them are detected. Therefore, if there are several unknown bacteria in the sample, it requires a large number of probes to detect how many other species are present. Complete characterisation of an unknown sample is therefore basically impossible. In these cases, PCR-DGGE can be a good complementary technique, as the number of bacteria in a sample can be detected on the basis of the number of bands in the gel. Also, with FISH and dot-blot it can be hard to get good signal intensities from the probes, especially from environmental samples with low bacterial activity.

CASE STUDIES

This article presents two different studies showing how specific bacterial strains can be followed over time by applying microbial tools. Even though both studies are related, degradation of halogenated organic compounds in bioreactors, the methods are general and can be applied to all bacterial systems.

Case study 1: strain stability in biological systems

Previous studies have shown that bioreactor systems that operate stably on a macroscopic scale can contain highly dynamic microscopic communities (Fernandez *et al*., 1999; Zumstein *et al*., 2000; Kaewpipat and Grady, 2002). It has also been shown that when specific strains are introduced into bioreactors, they can be overwhelmed (Massol-Deya *et al*., 1997; Bouchez *et al*., 2000; von Canstein *et al*., 2002). However, studies indicate that bioreactors fed on easily degradable substrates such as glucose and fructose contain highly dynamic cultures, while bioreactors fed on more hard to degrade substrates such as toluene, styrene and 2-dichloropropionic acid obtain more stable climax communities (Senior *et al*., 1976; Massol-Deya *et al*., 1997; Fernandez *et al*., 1999). However, to the authors' knowledge, no study has been performed on the stability of a single bacterial culture degrading a recalcitrant substrate.

Hence, the aim of Case Study 1 is to investigate (1) whether it is possible to operate a bioreactor under non-sterile conditions and maintain a stable dominant strain; (2) how sensitive this system would be to external disturbances; and (3) if stable strain/substrate systems exist, do they possess any general characteristics.

To answer these questions, two pure bacterial strains *Xanthobacter aut* GJ10 and *Bulkholderia sp* JS150

(hereafter referred to as only GJ10 and JS150, respectively) degrading two different toxic, recalcitrant organic substrates, 2-dichloroethane (DCE) and mono chlorobenzene (MCB), under non-sterile conditions were followed for 280 days.

Methods of case study 1

JS150 and GJ10 were inoculated into two continuously stirred tank reactors fed DCE and MCB, respectively. Flow rates and pH remained constant unless deliberately altered. Overall bioreactor performance was monitored by DCE and MCB removal, total organic content, chloride and CO_2 evolution. Strain stability was monitored by 16S rRNA by applying specific probes for GJ10 and JS150 and universal probes for all bacteria present. For a detailed description of the methods, see Emanuelsson *et al*. (2005).

Results of case study 1

Bioreactor functional stability

All bioreactor operational parameters apart from the dilution rate and pH (drop to 3.5 day 190 and 204) (DCE-degrading reactor only) were constant over time. Figure 1 shows the overall bioreactor stability for the DCE-degrading bioreactor. Constant carbon and chlorine evolution suggests functional stability, also evidenced by the almost complete DCE degradation. The bioreactor degrading MCB showed similar behaviour (results not shown). Furthermore, these macroscopic parameters remained relatively constant during the changes in the operating conditions reported above.

GJ10 strain stability

Figure 2 shows that the percentage of GJ10 remained at about 95% throughout the experiment. Figure 3 shows typical probing results for days 15 and 250, and it is clear from these that there was no change in the dominant strain. Also, to test the bacterial stability further, a large sample of industrial sludge was added to the bioreactor (50% of total bacteria after addition). However, after about 120 h, the percentage of GJ10 in the bioreactor was back to its value before the addition of the industrial sludge.

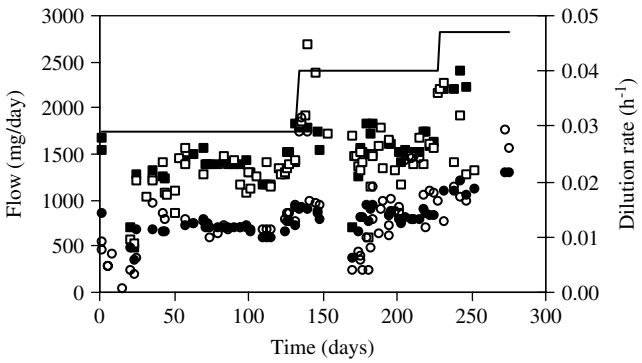

Figure 1. Chloride and carbon flows over time for the DCE-degrading bioreactor: ■ theoretical chloride evolution; □ chloride ion; ● carbon in; ○ carbon out as CO_2, biomass and TOC; ▬ dilution rate.

JS150 strain stability

In contrast to GJ10, the strain JS150 disappeared from the MCB-degrading bioreactor within a week of start-up (Fig. 4). Plating showed that at least eight strains were present after 475 days of bioreactor operation.

Discussion

To our knowledge, this is the first time it has been shown that one bacterial strain can remain nearly pure in a non-sterile bioreactor over a long time period. In

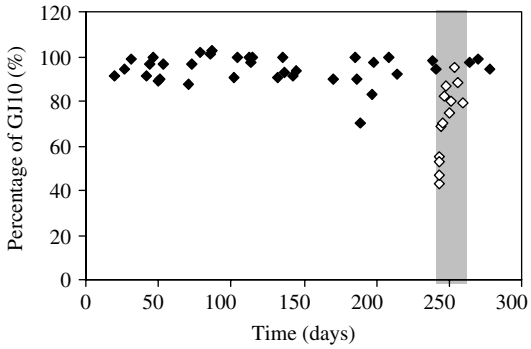

Figure 2. Percentage of GJ10 cells. Highlighted area shows the percentage of GJ10 during a sludge pulse injection.

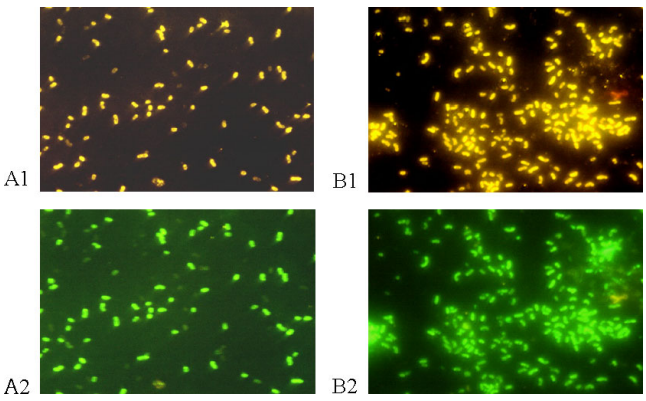

Figure 3. Epifluorescence photographs of hybridized bacteria: (A) field from day 15; (B) field from day 250. 1: cells hybridised with GJ10-specific probe; 2: cells hybridised with EUB338I probe. This figure is available in colour online at www.apjChemEng.com.

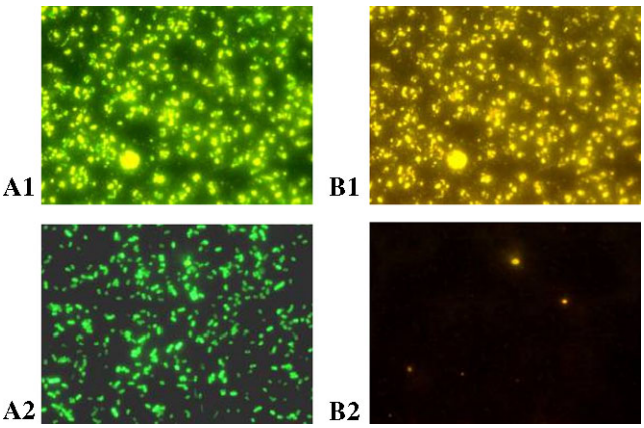

Figure 4. Epifluorescence photographs of hybridised bacteria (MCB). (A) field from day 4; (B) field from day 20. 1: cells hybridised with JS150 specific probe; 2: cells hybridised with EUB338I probe. This figure is available in colour online at www.apjChemEng.com.

addition, it remained stable despite external interference such as dilution rate, pH drops and contamination by foreign microorganisms. However, this is not a general result, as, in contrast, JS150 disappeared within a week of start-up.

The unusual stability of the GJ10 system is mainly thought to be due to the degradation pathway of DCE. Key genes involved in the DCE degradation are located only on the chromosome, while the genes involved in the MCB degradation are located on the plasmid (van der Ploeg *et al*., 1994; Kahng *et al*., 2001). Genes located on plasmids are easily transferred to other organisms, while genes on chromosomes are not (Dejonghe *et al*., 2002). Furthermore, there are few other microorganisms reported that are capable of degrading DCE, while it has been shown that the oxygenase enzyme cleaving the ring formation in the MCB degradation is similar to oxygenases in other bacteria and widely spread in nature (Johnson and Olsen, 1997). This makes it likely that the ability to degrade MCB can be readily transferred to other microorganisms and if their growth rate on MCB is faster than that of the JS150 bacteria, the JS150 will be rapidly out-competed, as observed.

Case study 2: biodegradation of 2-fluorobenzoate in up-flow fixed bed bioreactors (UFBR)

A drawback with nearly all biological treatment processes is the sensitivity of the bacteria to changes in substrate load and composition. This can result in periods of bacterial starvation and wash out, thereby decreasing the effectiveness of the treatment unit (Eichner *et al*., 1999). Furthermore, many compounds widely used in industry are toxic, carcinogenic and not readily biodegradable, such as fluorinated organic compounds. Hence, if the treatment system does not work properly, these compounds accumulate in the environment. Studies have found that immobilised bacteria remain more active under starvation periods and have shorter lag periods when fed on a compound that they can degrade (Nicolella *et al*., 2000).

Hence, to further improve the understanding of biodegradation of fluorinated organic compounds and bioreactor responses to alternating operational conditions, the aim of Case study 2 was to use an isolated bacterial culture, capable of degrading a model fluorinated organic compound (2-flourobenzoate, 2-FB), to study the biodegradation of 2-FB and to follow microbial community over time in three UFBRs inoculated with the degrading strain.

METHODS FOR CASE STUDY 2

A 2-FB-degrading strain (FB2) enriched from sediments of an industrially contaminated site in Portugal was used. Three different UFBRs, each containing polyethylene (PE), granular activated carbon (GAC) or expanded clay particles (EC) as growth supports, were established. The reactors were operated for almost seven months under dynamic conditions (0–270 ppm 2-FB and 6.5–14 h HRT). The microbial dynamics were followed by PCR-DGGE and plate counts. For further details see Emanuelsson *et al*. (2006).

Results

Bioreactor operation

Figure 5 shows the biodegradation for the EC-packed UFBR as an example. 2-FB was not detected in the

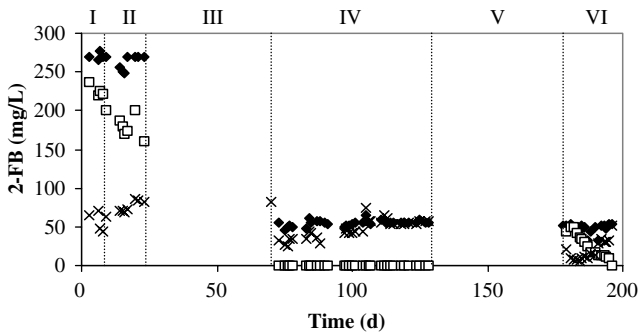

Figure 5. Degradation of 2-FB in the EC-packed bioreactor. Day 1–8 (I): 0.9–1.14 g day^{-1} L^{-1} 2-FB (shock load) (I); day 9–24 (II): 0.4–0.5 g day^{-1} L^{-1} (shock load), day 25–72 (III): inlet stopped (starvation period); day 73–128 (IV): 0.09–0.11 g day^{-1} L^{-1}; day 129–177 (V): inlet stopped (starvation period); day 178–196 (VI): 0.09–0.1 g day^{-1} L^{-1}. **x** 2-FB degraded; ♦ 2-FB in inlet; □ 2-FB in outlet.

outlet from the reactor with GAC at any time during the experiment; however, high amounts of 2-FB were detected at the outlet of the reactors containing PE and EC during the first 24 days. After the first starvation period, 2-FB was degraded straight away after restarting the feed in all three reactors and no 2-FB was detected at the outlet. During phase IV (continuous feeding), all three reactors showed stable performance with 100% removal efficiency. After the second starvation period, the reactor containing PE did not survive, the reactor containing EC showed 100% removal efficiency after 14 days and the reactor containing GAC showed 100% removal efficiency straight away.

DGGE

Figure 6 shows the DGGE profiles from the three different reactors. FB2 was present in PE and EC reactors throughout the operation although the intensity of the band varied. From the DGGE gel it was not possible to infer whether FB2 was present in the reactor containing GAC until the end of the operation.

For the PE and GAC reactors, the largest shift in microbial community occurred between T4 and T5, which was during a continuous feeding period, and smaller shifts were also noted during the second starvation period. For the EC reactor, the largest shift occurred between T5 and T6, which was during the second starvation period.

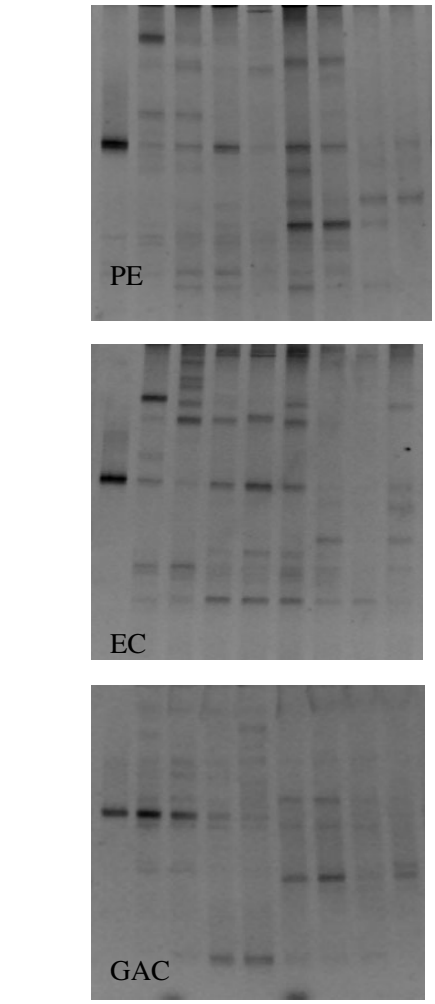

Figure 6. DGGE results for the three reactors. This figure is available in colour online at www.apjChemEng.com.

Discussion

This study confirmed the effectiveness of biofilm reactors, but also highlighted the importance of the type of materials used. The presence of GAC in the bioreactor improved the long-term performance and robustness of the reactor. The DGGE analysis showed that the reactors were quickly colonised by several bacterial strains but thereafter the bacterial communities were kept fairly stable, although starvation periods induced shifts in the microbial populations.

CONCLUSIONS

This paper has shown that it is relatively easy to get a more fundamental understanding of bioreactor performance by using molecular techniques to monitor the microbial population inside a treatment unit. This way, it is possible to follow long-term stability in communities as well as the activity and abundance of specific species. This can be used to understand the performance of the reactor better and provides clues to explain bioreactor failures. At the same time, this could help develop more robust start-up, feeding and bio-augmentation strategies.

Acknowledgment

This work was supported in part by the European Community's Human Potential Programme under contract HP-RTH-CT-2002-00213 (BIOSAP).

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